Research Article

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Relationship between *Salmonella enterica* attachment and leaf hydrophobicity, roughness, and epicuticular waxes: a focus on 30 baby-leaf salads

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Abstract

BACKGROUND: The first step in the contamination of leafy vegetables by human pathogens is their attachment to the leaf surface. The success of this is influenced strongly by the physical and chemical characteristics of the surface itself (number and size of stomata, presence of trichomes and veins, epicuticular waxes, hydrophobicity, etc.). This study evaluated the attachment of *Salmonella enterica* to 30 baby-leaf salads and tested whether the differences found among them were related to the following leaf traits: hydrophobicity, roughness, and epicuticular waxes.

RESULTS: Differences in susceptibility to contamination by *S. enterica* were found between the 30 baby-leaf salads investigated. The lowest attachment was found in wild lettuce (*Lactuca serriola* L.) and lamb's lettuce 'Trophy F1' (*Valerianella locusta* [L.] Laterr.), with values of 1.63 $\pm$ 0.39 Log(CFU/cm^2) and 1.79 $\pm$ 0.54 Log(CFU/cm^2), respectively. Attachment was correlated with hydrophobicity (measured as contact angle) ($r = -0.39$) and epicuticular waxes ($r = -0.81$) but not with roughness ($r = 0.24$). The most important wax components for attachment were alcohols and, in particular, the three-dimensional (3D) wax crystals of C26 alcohol, but fatty acids probably also had a role. Both these compounds increased hydrophobicity. The presence of thymol, whose antimicrobial properties are well known, was found in lamb's lettuce.

CONCLUSIONS: The findings of this study can help to predict and control the attachment and contamination of leafy salads by enterobacteria. They also provide useful information for breeding programs aiming to develop cultivars that are less susceptible to human pathogens, enhancing the food safety of vegetables.

Keywords: leafy vegetables; human pathogens; leaf surface; contact angle; epicuticular waxes; 3D wax crystals

INTRODUCTION

Human pathogens have conventionally been associated with foods originating from animal sources. Nevertheless, in recent decades, several outbreaks associated with fruit and vegetables have occurred, showing that plants are an important vector of foodborne diseases.[1] Contamination frequently takes place when crop plants encounter biologically polluted irrigation, wastewater, contaminated fertilizers, small carrier animals, and poor hygiene practices, before or after the harvest.[2,3] According to the European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC) 2021 zoonoses report,[1] the frequency distribution of strong-evidence foodborne outbreaks by food vehicles showed that vegetables and juices (and similar products) caused a higher number of outbreaks than traditional high-risk broiler meat. The same report listed *Salmonella* associated with vegetables and juices in the seventh position in the ranking of the top-ten pathogen/food pairs, with strong

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evidence that this caused 11 outbreaks in the EU in 2021.[1] Baby-leaf vegetables are particularly susceptible to contamination because of their tender leaves (high water content) associated with the early growth stage, and their use in salads, which involves eating them raw.[4] Several examples of foodborne disease outbreaks occurring from 2000 to 2016 linked to the combination of *Salmonella* and leafy greens eaten raw, specifically including baby leaves, are reported by Mogren *et al*.[5] Another case in 2021 concerned a multistate *Salmonella* outbreak in the USA linked to packaged baby-leaf salads, as described by the US Centers for Disease Control and Prevention (CDC).[6]

The first step in the contamination of salads with bacteria is attachment to the leaf surface, which, in the absence of timely decontamination treatments, can be followed by internalization.[7,8] The interaction between bacteria and leaves is a complex process influenced by various factors, including the physical and chemical characteristics of the leaf surface.[9] Previous research has shown that the leaf surface plays a critical role in providing attachment sites for human pathogens such as *Salmonella enterica* and *Escherichia coli*,[10] and influences the establishment of bacterial communities on the leaves.[11,12] Surface roughness, in particular, can provide numerous microhabitats for bacteria to attach and hide, offering protection from external factors such as wind, rain, and sunlight, including protection from UV radiation.[10,12,13,14] In spinach, leaf-blade roughness and stomata density influenced the persistence of *E. coli* O157:H7.[14] Trichomes, stomata, and leaf veins also contribute to overall roughness. Doan *et al*. (2020)[10] showed that leaf venation prevented the recovery of *E. coli* from the surface of spinach leaves using water washing and rinsing, even in the presence of a detergent, and increased the ability of the bacteria to survive chlorine washing.

Another factor involved in bacterial attachment is the hydrophobic or hydrophilic nature of the leaf surface. In general, bacteria are more likely to attach to hydrophilic surfaces due to favorable interactions with water molecules and surface charges.[15] The presence of epicuticular waxes (waxes of the outermost layer of the leaf surface) makes leaves hydrophobic to an extent, dependent on their amount and chemical composition, thus influencing the attachment of human pathogens.[16,17]

The aims of this study were: (i) to evaluate the attachment of *S. enterica* on the leaves of 30 green salads contaminated at the baby-leaf stage; (ii) to test whether the differences in susceptibility to *Salmonella* contamination among the salads were related to the following leaf traits: hydrophobicity, roughness, and epicuticular waxes. Leaf water status was also considered. It is important to know these relationships not only in order to understand plant-bacteria interactions but also to provide information that could possibly be useful to improve the safety of baby leaves in a farm-to-fork scenario.

MATERIALS AND METHODS

Production of the baby leaves

Baby leaves of 30 different accessions belonging to 13 species were tested. Seeds of the 30 accessions were used as starting material. The detailed list of the accessions and the seed source are reported in Truschi *et al*. (2023).[12] The same article[12] describes the cultivation method: seeds were sown in polystyrene alveolate trays filled with vermiculite at a density of 3000 seeds m^{-2}. After sowing, trays were kept for 48 h in the dark at 20 °C for promoting germination and were then transferred in a floating system in a growth chamber at 21 ± 2 °C (day) and 14 ± 2 °C (night) with a photoperiod of 16 h under fluorescent lighting units OSRAM L36W/77 (36 W, 120 cm in length, 26 mm in diameter) (OSRAM Beteiligungen GmbH, Munich, Germany). A full-strength Hoagland's nutrient solution (macroelements expressed in mM and microelements in μM: N 15.0, P 0.10, K 6.0, Ca 5.0, Mg 2.0, Fe 50.0, B 46.2, Mn 9.2, Zn 0.78, Cu 0.32, Mo 0.12) was used. At the baby-leaf stage (5/6 weeks after sowing depending on the species) plants were cut at the base of the petiole and immediately inoculated. The following leaf parameters were also analyzed: hydrophobicity, roughness, and wax and water content (see the following paragraphs). Water content was calculated using the formula $[(FW - DW)/FW] \times 100$, where FW is fresh weight and DW is dry weight measured after oven drying at 80 °C until constant weight.[12]

Salmonella enterica surface inoculation of the baby leaves

Salmonella enterica subsp. *enterica* serovar Typhimurium ATCC 19585 was used for the surface inoculation.

The inocula started from a −20 °C glycerol stock. Briefly, 1 mL from an overnight inoculum in lysogeny broth (LB) (Oxoid, Basingstoke, UK) was washed three times in a sterile physiological solution (PS) (NaCl 0.85% w/v in H_2O; Oxoid) to clean the cells and remove any residual LB medium. The working bacterial suspension was prepared by further diluting the washed cells to a 1:10 ratio in PS and the optical density at 600 nm (OD600) was adjusted to 0.1 ± 0.015 absorbance. This resulted in a working bacterial suspension with an approximate concentration of 5×10^7 cells per milliliter. Leaf disks 1.3 cm in diameter were cut from leaves of different plants. Twenty microliters of the working bacterial suspension were placed onto the center of the leaf disks on the adaxial side and incubated for 5 min at 25 °C in static condition. After incubation, the leaf disks were gently picked up with sterile tweezers and washed four times in 15 mL of clean PS in glass tubes to remove unattached bacterial cells. Rinsed disks were subsequently ground with a mini-pestle in 0.5 mL of PS into 1.5 mL tubes. After grinding, 20 μL of the suspension was plated onto selective and differential medium Xylose Lysine Desoxycholate Agar (XLD agar; Oxoid) and incubated at 37 °C overnight. Colony-forming units (CFU) were counted and log base 10 (lg) transformed.

Contact angle measurement

The hydrophobicity of the leaf surface of the 30 accessions was quantified by measuring the contact angle using a drop shape analyzer equipped with a video camera and connected to a computer (DSA 25E; Krüss, Hamburg, Germany).[18] Leaf samples collected from different plants were carefully placed on clean microscopic slides using double-sided adhesive tape. Droplets of pure water (10 μL) were placed carefully on the adaxial side of the leaf surfaces. Each droplet equilibrated 10 s on the surface before measurement. Contact angles were measured using the sessile drop method. This method determines the contact angle from the shadow image of a sessile drop and is based on an ellipse algorithm (tangent^{-1}). Each measurement was done with a fresh-water droplet.

Leaf surface roughness

A portable three-dimensional (3D) digital microscope used for imaging the leaf surface and measuring local roughness was based on the 'deep focus' technique.[19] The operating principle is based on the simple observation that, due to the limited depth of focus of each optical system, only objects placed at a suitable distance from the sensor form in-focus images, whereas those located at different distances appear out of focus (blurred). Thus, to reconstruct the surface of an object, a series of images of the same scene are acquired, corresponding to different positions of the optical group (including imaging optics, digital camera, and the smart lighting system),[20-22] translated along the optical axis. Each image will contain in-focus and out-of-focus parts of the surface under examination. Dedicated software processes the sequence of images. From each image, the focused areas are extracted, and, to reconstruct the 3D surface, combined together to obtain the depth scale when the translation is provided. In this study, the calibrated translation stage had a minimum step size in the micron range, resulting in a field of view of approximately 7 × 5.2 mm and a vertical resolution of approximately 10 μm. Surface roughness parameters were extracted from 3D reconstruction, according to ISO standards,[23] focusing the analysis of the plant surface on the average roughness (Ra). These are completely non-contact and non-destructive measurements, which overcomes the disadvantages of using a contact stylus profilometer with soft biological samples.

Quantification and qualification of epicuticular waxes

The protocol described by Baales *et al.*[24] was used for the quantification and identification of individual wax components. Glass vials with broad rims and a central opening with a defined area were filled with chloroform (1.5 mL). Intact leaves of different plants were placed carefully on a clean Teflon disk. Due to the different size of the leaves, two defined areas were used (0.384 and 1.25 cm^2).

The leaf side of interest was pressed gently on the opening of the glass vial and turned upside down for 10 s to allow wax extraction by chloroform in the vial. Subsequently, the wax extract was spiked directly with the C_{24} alkane (internal standard) and the volume was reduced to 200 μL under a gentle stream of nitrogen at 60 °C. Prior to gas chromatography, samples were derivatized using *N*, *O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) (Merck, Darmstadt, Germany) at 70 °C for 45 min. For derivatization, 20 μL BSTFA and 20 μL pyridine as a catalyst were added to the samples dissolved in 200 μL of chloroform. Quantification was performed by on-column injection analyzing 1 μL of each sample in a gas chromatograph connected to a flame ionization detector (GC-FID) (Agilent 5980; column: 30 m DB-1 with an inner diameter of 0.32 mm and film 0.1 μm; Agilent Technology, Santa Barbara, CA, US). Identification of wax was achieved by gas chromatography-mass spectrometry (GC-MS) (Agilent 6890 N; MS: Agilent 5973 N mass selective detector; column: 30 m DB-1MS with an inner diameter of 0.32 mm and film 0.1 μm; Agilent Technology). Identification of the individual peaks was based on fragmentation patterns of the peaks and by comparing the mass spectra that were obtained with stored mass spectra in the National Institute of Standards and Technology (NIST) 2011 library.

Moreover, images of the epicuticular wax crystals were taken using a scanning electron microscope (SEM XL20; Philips, Amsterdam, Netherlands). The images were captured under low vacuum at 10 kV and 800× and 8000× resolution from at least three different samples per accession.

Statistical analysis

All the statistical analyses were performed using Rstudio software[25] (version 4.3.1). The experimental design included three trays per accession, with about 84 plants per tray. A different number of plants was randomly collected from the trays for analyzing the different parameters: 12 plants for water content, five for *S. enterica* inoculation, 8–12 for roughness, five for contact angle, and three for wax concentration. Data were subjected to the Shapiro–Wilk test for normality and Levene's test for homogeneity of variances to verify the assumptions of the analysis of variance (ANOVA), using the *car* package.[26] As the assumptions were not respected, a linear mixed model (LMM) was applied to all the parameters, considering the accessions as fixed factors and the repeated measures as random factors, using the *lme4* package.[27] Then, the Tukey test was applied ($P \leq 0.05$) using the *multcompView* package.[28] Pearson's correlation test ($P \leq 0.05$) was also used to determine the relationship between *S. enterica* ATCC 19585 attachment and the leaf traits being considered, using the *bruceR* package.[29] Both total wax concentration and its components fatty acids and alcohols (and, among the latter, C26 alcohol) were considered in the correlation test, and the compounds detected in only a small number (1–7) of accessions (aldehydes, alkanes and esters) were excluded. A principal component analysis (PCA) was carried out using R Studio with *FactorMineR* and *Factorextra* packages for all recorded data.[30,31] The highly correlated variables ($r > 0.90$) were excluded from the PCA. Finally, a partial least squares (PLS) model was established to predict the amount of *S. enterica* attachment (Y variable) by specifying six variables (X variables: contact angle, surface roughness, C26 alcohol, alcohols, fatty acids, and water content) using the *mdatools* package.[32] Leave-one-out cross-validation (LOOCV), using the coefficient of determination (R^2), and the root mean square error of prediction (RMSE) were applied to verify the PLS model. Parameters that had variable importance for projection (VIP) <0.8 were not considered to make a major contribution to dimensionality reduction in PLS.

RESULTS

Salmonella enterica attachment in baby leaves

Significant differences in *S. enterica* attachment ($P < 0.001$) were observed among the 30 baby-leaf accessions (Table 1). Sorrel, Red Giant leaf mustard, pak-choi, rocket, endive, Swiss chard, and mizuna showed a contamination level higher than 3.7 log CFU/cm^2 and were found to be significantly more susceptible to *Salmonella* contamination than wild lettuce, lamb's lettuce, wild rocket 'Yeti', lettuce 'Pamela', Lollo Rossa lettuce, dandelion Ingegnoli, wild rocket Ingegnoli, and blonde lettuce (from 1.63 to 3.29 log CFU/cm^2). Intermediate values (from 3.39 to 3.66 log CFU/cm^2) were measured in wild chicory Ingegnoli, romaine lettuce 'Maraichere', chicories 'Magdeburgo' and 'Spadona da Taglio', wild chicories (B&T and local), spinach 'Cugoe RZ F1', romaine 'Bionda degli Ortolani', red-leaf mustard, local dandelion, Lollo Verde lettuce, red chard 'Bull's Blood Artica', chicories witloof, 'Biondissima di Trieste', and mizuna. Lamb's lettuce 'Trophy F1' and wild lettuce were significantly different from all the other accessions, with the lowest level of contamination (1.79 ± 0.54 and 1.63 log CFU/cm^2, respectively).

Contact angle

Significant differences in hydrophobicity ($P < 0.001$) were found between the 30 accessions. Wild lettuce leaves showed the highest contact angle (136.5 ± 6.97°), and were different from all the other accessions, with the worst wettability. On the other hand, chicory 'Spadona da Taglio' had the smallest contact angle (28.49 ± 5.75°) but was not significantly different from other chicories ('Biondissima di Trieste' and witloof) or Lollo Rossa lettuce (Table 1).

Table 1. *Salmonella enterica* attachment (log(CFU/cm^2)), contact angle (°), leaf roughness (Ra), and leaf water content (%), in the 30 baby-leaf accessions (ID = accession identification)

ID[a]	Botanical family	Species	Accession	Attachment log(CFU/cm^2)	Contact angle (°)	Leaf roughness (Ra)	Leaf water content (%)
7	Asteraceae	*Cichorium endivia* L.	Endive	3.72 ± 0.01 a	46.76 ± 5.78 gh	13.88 ± 0.97 ef	91.40 ± 3.31 ab
6		*Cichorium intybus* L.	Chicory 'Biondissima di Trieste'	3.59 ± 0.02 abc	40.38 ± 3.80 hi	18.09 ± 3.55 cdef	93.65 ± 0.94 a
2			Chicory 'Magdeburgo'	3.42 ± 0.01 abcde	55.76 ± 1.07 fgh	13.89 ± 3.93 ef	92.77 ± 1.36 a
22			Chicory 'Spadona da Taglio'	3.44 ± 0.02 abcde	28.57 ± 7.04 i	31.34 ± 9.39 abcdef	92.18 ± 0.55 ab
5			Wild chicory (B&T)	3.42 ± 0.01 abcde	45.12 ± 8.70 gh	43.83 ± 11.16 ab	91.64 ± 0.82 ab
1			Wild chicory (Ingegnoli)	3.39 ± 0.01 abcdef	52.14 ± 5.41 gh	26.50 ± 3.85 abcdef	93.56 ± 2.79 a
4			Wild chicory (local)	3.44 ± 0.07 abcde	45.72 ± 6.82 gh	15.86 ± 2.47 def	90.68 ± 3.54 ab
3			Witloof chicory	3.62 ± 0.1 abc	39.62 ± 2.15 hi	14.84 ± 2.19 ef	92.46 ± 1.54 a
29		*Lactuca sativa* L.	Blonde lettuce	3.29 ± 0.03 bcdef	51.09 ± 3.87 gh	23.91 ± 2.95 abcdef	96.11 ± 0.83 a
28			Lettuce 'Pamela'	3.11 ± 0.02 ef	48.68 ± 2.10 gh	41.88 ± 12.51 abc	93.30 ± 1.31 a
9			Lollo Rossa lettuce	3.17 ± 0.03 def	44.35 ± 2.38 ghi	27.77 ± 3.19 abcdef	94.78 ± 0.97 a
8			Lollo Verde lettuce	3.49 ± 0.03 abcde	58.12 ± 8.81 fg	38.13 ± 8.29 abcde	95.75 ± 0.20 a
23			Romaine lettuce 'Bionda degli Ortolani'	3.46 ± 0.08 abcde	48.62 ± 4.54 gh	24.00 ± 2.65 abcdef	95.10 ± 0.65 a
30			Romaine lettuce 'Maraichere'	3.40 ± 0.03 abcde	92.28 ± 1.09 cd	19.04 ± 5.11 bcdef	92.89 ± 1.56 a
33		*Lactuca serriola* L.	Wild lettuce	1.63 ± 0.39 g	135.54 ± 5.52 a	20.73 ± 5.98 bcdef	90.31 ± 0.55 ab
15		*Taraxacum officinale* L.	Dandelion (Ingegnoli)	3.22 ± 0.02 cdef	55.34 ± 1.74 fgh	12.18 ± 1.30 f	89.59 ± 0.49 ab
14			Dandelion (local)	3.49 ± 0.02 abcde	50.27 ± 1.40 gh	13.95 ± 1.79 ef	85.19 ± 9.61 b
20	Brassicaceae	*Brassica juncea [L.] Czern.*	Red Giant leaf mustard	3.77 ± 0.01 a	71.34 ± 1.85 ef	46.80 ± 15.43 a	94.04 ± 0.52 a
16			Red leaf mustard	3.49 ± 0.03 abcde	85.77 ± 6.80 de	19.68 ± 2.54 bcdef	93.08 ± 0.56 a
25			Wasabina leaf mustard	3.76 ± 0.01 a	70.94 ± 7.10 ef	20.69 ± 4.54 bcdef	93.72 ± 0.46 a
19		*Brassica rapa* L.	Mizuna	3.66 ± 0.09 ab	104.76 ± 2.52 bc	19.72 ± 9.71 bcdef	93.41 ± 0.83 a
17			Pak-choi	3.75 ± 0.01 a	85.37 ± 5.36 de	40.29 ± 14.21 abcd	95.49 ± 0.29 a
32		*Diplotaxis tenuifolia [L.] D.C.*	Wild rocket 'Yeti'	2.98 ± 0.09 f	109.72 ± 1.83 b	18.38 ± 6.34 cdef	92.66 ± 0.55 a
24			Wild rocket (Ingegnoli)	3.25 ± 0.06 bcdef	108.92 ± 2.75 b	28.19 ± 11.09 abcdef	93.95 ± 1.04 a
10		*Eruca vesicaria [L.] Cav. subsp. sativa [Mill.] Thell*	Rocket	3.73 ± 0.01 a	92.27 ± 3.55 cd	28.08 ± 9.23 abcdef	93.33 ± 1.53 a
31	Chenopodiaceae	*Beta vulgaris* L.	Red chard 'Bull's Blood Artica'	3.56 ± 0.02 abcd	97.78 ± 7.49 bcd	21.07 ± 2.35 bcdef	93.38 ± 1.51 a
27			Swiss chard	3.72 ± 0.03 a	90.27 ± 2.22 cd	25.89 ± 10.52 abcdef	93.19 ± 2.12 a
26		*Spinacia oleracea* L.	Spinach 'Cugoe RZ F1'	3.45 ± 0.01 abcde	88.46 ± 7.24 d	29.24 ± 17.84 abcdef	93.29 ± 0.28 a
21	Polygonaceae	*Rumex acetosa* L.	Sorrel	3.79 ± 0.04 a	46.19 ± 5.53 gh	21.98 ± 1.50 abcdef	93.04 ± 1.23 a
12	Valerianaceae	*Valerianella locusta [L.] Laterr.*	Lamb's lettuce 'Trophy F1'	1.79 ± 0.54 g	82.39 ± 7.25 de	12.87 ± 3.07 f	89.92 ± 1.76 ab

Data are means ± SDs. In the same column, different letters show statistically significant differences for $P < 0.001$ (Tukey test).
[a] Accession identification number.

Roughness and water content

Three-dimensional digital microscopy revealed significant differences ($P < 0.001$) for the Ra parameter among the 30 accessions. As shown in Table 1, the smoothest leaf surfaces (lowest Ra values) were found in lamb's lettuce 'Trophy F1' and dandelion Ingegnoli, with values (12.87 ± 3.07 μm and 12.18 ± 1.30 μm, respectively) significantly different from wild chicory B&T, the lettuces 'Pamela' and Lollo Verde, pak-choi, and Red Giant leaf mustard. The latter accession had the roughest leaves (46.80 ± 15.43 μm). From a purely visual point of view, the difference between lamb's lettuce and Red Giant leaf mustard can be observed in the 3D reconstructions of the leaf surface shown in Fig. 1. A rough estimate of the different degrees of roughness can be obtained by observing the height difference between the bulges and cavities (expressed by the scale of colors).

All the baby-leaf salads but the two dandelion accessions had a water content higher than 90%. In some lettuces (blonde, Lollo Verde, and romaine 'Bionda degli Ortolani') and in pak-choi it was even higher than 95% (Table 1). Local dandelion showed the lowest value (85.19%), significantly different ($P < 0.001$) from the species *Lactuca sativa*, *Cichorium intybus* accessions 'Biondissima di Trieste', 'Magdeburgo', wild Ingegnoli, and witloof, *Brassica juncea*, *Brassica rapa*, *Diplotaxis tenuifolia*, *Eruca vesicaria*, *Beta vulgaris*, *Spinacia oleracea*, and *Rumex acetosa* (Table 1).

Wax quantification and characterization

The 30 baby-leaf salads also differed significantly in the total amount of wax ($P < 0.001$). In all the accessions the epicuticular waxes included fatty acids and alcohols, whereas aldehydes, alkanes, and esters were only found in a limited number of accessions (Table 2). Only wild lettuce showed aldehydes (0.82 ± 0.08 μg/cm^2); alkanes were detected in spinach 'Cugoe RZ F1' (0.69 ± 0.16 μg/cm^2), wild lettuce (0.20 ± 0.04 μg/cm^2), and sorrel (0.07 ± 0.01 μg/cm^2); whereas esters in the lettuce group (*L. sativa*) and wild lettuce (*L. serriola*) had values ranging from 0.07 to 0.79 ± 0.02 μg/cm^2. Alcohols were the main component of waxes (about 80% of the total waxes as an average of the 30 accessions). Only in rocket and wild rocket did the fatty acid content exceed that of alcohol (Table 2). Both the wild rockets had significantly higher fatty acid amounts (2.66 ± 0.45 μg/cm^2 and 2.05 ± 0.89 μg/cm^2, respectively) than all the other accessions. In most chicories (local wild chicory: 0.04 ± 0.02 μg/cm^2, 'Biondissima di Trieste': 0.05 ± 0.02 μg/cm^2, witloof: 0.05 ± 0.02 μg/cm^2, wild chicory B&T: 0.06 ± 0.00 μg/cm^2, 'Magdeburgo': 0.06 ± 0.01 μg/cm^2), as well as in local dandelion (0.07 ± 0.02 μg/cm^2) and Wasabina leaf mustard (0.09 ± 0.01 μg/cm^2) fatty acids concentrations were significantly lower than in Red leaf mustard (0.77 ± 0.24 μg/cm^2) and rocket (0.75 ± 0.25 μg/cm^2) (Table 2). Wild lettuce was by far the accession with the highest alcohol content (12.32 ± 1.35 μg/cm^2), of which 84.3% was C26 alcohol. Wild lettuce was followed by 'Pamela' lettuce, Lollo Rossa lettuce, lamb's lettuce 'Trophy F1', and 'Maraichere' romaine lettuce, with 3.34 ± 1.08 μg/cm^2, 3.02 ± 0.70 μg/cm^2, 2.87 ± 0.88 μg/cm^2, and 2.76 ± 0.39 μg/cm^2 alcohol concentration, respectively. These values were significantly higher than those of a large group of 13 accessions ranging from 1.43 ± 0.03 μg/cm^2 (witloof chicory) to 0.34 ± 0.07 μg/cm^2 (sorrel). In the four accessions mentioned above, the C26 alcohol accounted for 8% to 22% of the total alcohols. Higher percentages of this component were observed in other accessions (e.g., 55% in Wasabina leaf mustard), but with low absolute values. Alcohols were highly correlated with total wax content (Fig. 2). Wild lettuce and sorrel were consistently the accessions with the highest (14.23 ± 3.60 μg/cm^2) and the lowest (0.51 ± 0.09 μg/cm^2) total amounts of wax, respectively, as can be seen in SEM images (Fig. 3). In lamb's lettuce, the gas chromatographic analysis also revealed the presence of thymol, a monoterpenoid phenol, in amounts of 0.26 ± 0.07 μg/cm^2 (data not shown).

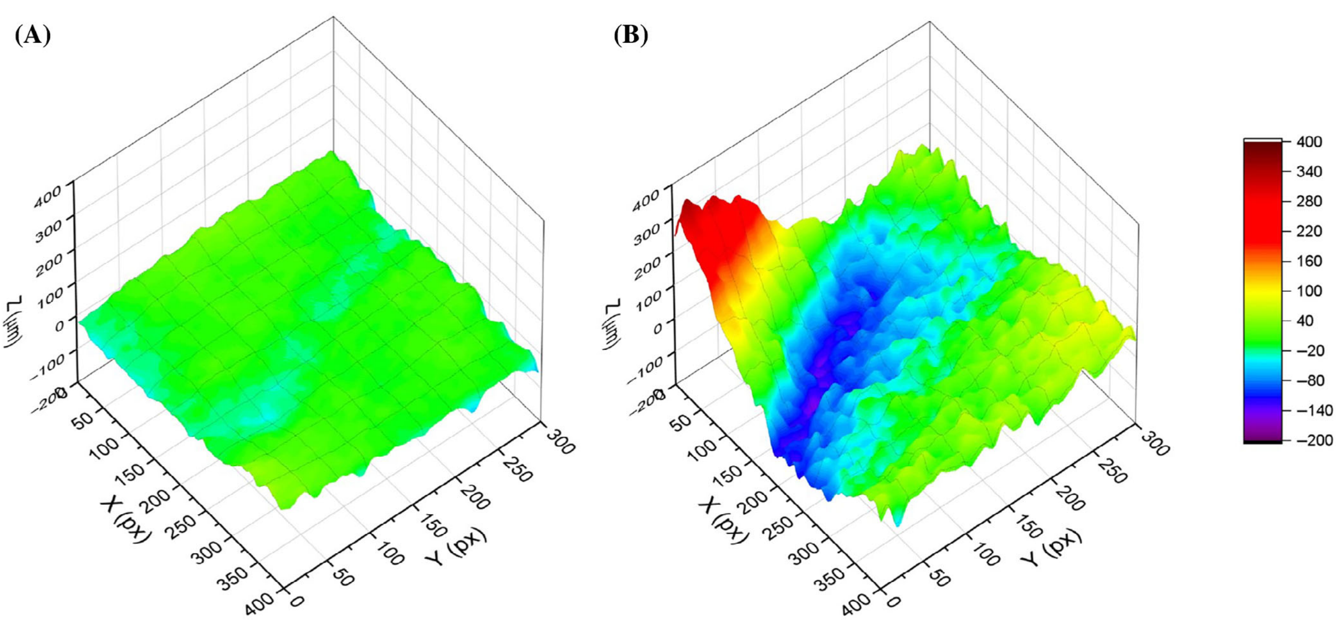

Figure 1. Examples of leaf surface three-dimensional (3D) reconstructions: lamb's lettuce 'Trophy F1' (A) and Red Giant leaf mustard (B). *X* and *Y* axes are expressed in pixels (1 pixel = 17.5 μm), with heights reported on the vertical axis (μm). Observing the height differences between bulges and cavities (expressed by the scale of colors) it is possible to obtain a rough estimate of the different degrees of roughness. The color ramp is shown on the right side of the figure.

Table 2. Chemical composition of epicuticular waxes in the 30 baby-leaf accessions

ID[a]	Accession	Fatty acid (μg/cm^2)	Alcohol (μg/cm^2)	Aldehyde (μg/cm^2)	Alkane (μg/cm^2)	Ester (μg/cm^2)	Total wax (μg/cm^2)
7	Endive	0.10 ± 0.05 cd	0.77 ± 0.20 hijk	-	-	-	0.87 ± 0.16 cde
6	Chicory 'Biondissima di Trieste'	0.05 ± 0.02 d	1.30 ± 0.20 fghijk	-	-	-	1.34 ± 0.18 bcde
2	Chicory 'Magdeburgo'	0.06 ± 0.01 d	1.77 ± 0.03 cdefghij	-	-	-	1.83 ± 0.11 bcde
22	Chicory 'Spadona da Taglio'	0.19 ± 0.1 bcd	1.81 ± 0.21 cdefghij	-	-	-	1.99 ± 0.30 bcde
5	Wild chicory (B&T)	0.06 ± 0.00 d	2.17 ± 0.23 bcdefgh	-	-	-	2.23 ± 0.23 bcde
1	Wild chicory (Ingegnoli)	0.23 ± 0.03 bcd	2.11 ± 0.44 bcdefghi	-	-	-	2.34 ± 0.41 bcde
4	Wild chicory (local)	0.04 ± 0.02 d	1.59 ± 0.22 cdefghijk	-	-	-	1.63 ± 0.22 bcde
3	Witloof chicory	0.05 ± 0.02 d	1.43 ± 0.03 efghijk	-	-	-	1.48 ± 0.05 bcde
29	Blonde lettuce	0.17 ± 0.07 bcd	2.39 ± 0.16 bcdef	-	-	0.09 ± 0.03 c	2.65 ± 0.14 bcde
28	Lettuce 'Pamela'	0.18 ± 0.03 bcd	3.34 ± 1.08 b	-	-	0.07 ± 0.00 c	3.59 ± 1.05 b
9	Lollo Rossa lettuce	0.17 ± 0.02 bcd	3.02 ± 0.70 bc	-	-	0.16 ± 0.02 b	3.34 ± 0.72 bc
8	Lollo Verde lettuce	0.16 ± 0.03 bcd	1.52 ± 0.29 defghijk	-	-	0.14 ± 0.01 b	1.82 ± 0.02 bcde
23	Romaine lettuce 'Bionda degli Ortolani'	0.26 ± 0.03 bcd	1.70 ± 0.07 cdefghijk	-	-	0.16 ± 0.04 b	2.12 ± 0.20 bcde
30	Romaine lettuce 'Maraichere'	0.14 ± 0.05 bcd	2.76 ± 0.39 bcde	-	-	0.09 ± 0.01 c	2.99 ± 0.43 bcde
33	Wild lettuce	0.10 ± 0.05 cd	12.32 ± 1.35 a	0.82 ± 0.08	0.20 ± 0.04 b	0.79 ± 0.02 a	14.23 ± 3.60 a
15	Dandelion (Ingegnoli)	0.12 ± 0.07 cd	2.36 ± 0.31 bcdefg	-	-	-	2.47 ± 0.38 bcde
14	Dandelion (local)	0.07 ± 0.02 d	1.70 ± 0.21 cdefghijk	-	-	-	1.77 ± 0.20 bcde
20	Red Giant leaf mustard	0.50 ± 0.01 bcd	0.50 ± 0.14 jk	-	-	-	0.55 ± 0.14 de
16	Red leaf mustard	0.77 ± 0.24 b	0.75 ± 0.19 hijk	-	-	-	1.52 ± 0.61 bcde
25	Wasabina leaf mustard	0.09 ± 0.01 d	0.44 ± 0.11 jk	-	-	-	0.53 ± 0.11 de
19	Mizuna	0.37 ± 0.19 bcd	0.61 ± 0.32 jk	-	-	-	0.98 ± 0.5 bcde
17	Pak-choi	0.28 ± 0.12 bcd	0.59 ± 0.32 jk	-	-	-	0.87 ± 0.44 cde
32	Wild rocket 'Yeti'	2.66 ± 0.45 a	0.71 ± 0.09 ijk	-	-	-	3.37 ± 0.77 bc
24	Wild rocket (Ingegnoli)	2.05 ± 0.89 a	0.93 ± 0.66 ghijk	-	-	-	2.98 ± 1.34 bcde
10	Rocket	0.75 ± 0.25 bc	0.50 ± 0.13 jk	-	-	-	1.25 ± 0.35 bcde
31	Red chard 'Bull's Blood Artica'	0.17 ± 0.04 bcd	1.64 ± 0.20 cdefghijk	-	-	-	1.81 ± 0.24 bcde
27	Swiss chard	0.14 ± 0.05 bcd	1.61 ± 0.23 cdefghijk	-	-	-	1.75 ± 0.23 bcde
26	Spinach 'Cugoe RZ F1'	0.46 ± 0.14 bcd	0.92 ± 0.19 hijk	-	0.69 ± 0.16 a	-	2.06 ± 0.46 bcde
21	Sorrel	0.11 ± 0.01 cd	0.34 ± 0.07 k	-	0.07 ± 0.01 c	-	0.51 ± 0.09 e
12	Lamb's lettuce 'Trophy F1'	0.27 ± 0.05 bcd	2.87 ± 0.88 bcd	-	-	-	3.14 ± 0.93 bcd

Data are means ± SD. In the same column, different letters show statistically significant differences for $P < 0.001$ (Tukey test).
[a] Accession identification number.

Correlation between *S. enterica* attachment and leaf hydrophobicity, roughness, and wax content

The results of the Pearson's correlation test are shown in Fig. 3. A significant negative correlation was observed between the *S. enterica* attachment and total waxes ($r = -0.81$; $P < 0.001$), its alcoholic component ($r = -0.78$; $P < 0.001$) and in particular C26 alcohol ($r = -0.66$; $P < 0.001$), and the contact angle ($r = -0.39$; $P < 0.05$). The latter parameter was positively correlated with total waxes ($r = 0.48$; $P < 0.01$), fatty acids ($r = 0.50$; $P < 0.01$), and C26 alcohol ($r = 0.48$; $P < 0.01$). As shown in Fig. 3, roughness and water content were slightly correlated ($r = 0.50$; $P < 0.05$). The total wax amount was positively correlated with both alcohols ($r = 0.97$; $P < 0.001$) and C26 alcohol ($r = 0.94$; $P < 0.001$), which were correlated with each other ($r = 0.94$; $P < 0.001$).

Principal component analysis results

The first principal component (Dim1) of the PCA analysis explained 44.7% of the total variation among the accessions (Fig. 4). The major parameters contributing to Dim1 were total waxes, *S. enterica* attachment, and alcohols (0.89, 0.81, and 0.80, respectively). The second component (Dim2) explained 21% of the total variation, with fatty acids providing the major contribution, followed by contact angle (0.71 and 0.48, respectively) (Fig. 4(A)). When grouped according to their botanical family, wild lettuce (accession 33) stood out within the Asteraceae for its high alcohol and wax content and its low contamination level (Fig. 4(A), (B)). Lamb's lettuce 'Trophy F1' (Valerianaceae, accession 12) was close to wild lettuce, and sorrel (Polygonaceae, accession 21), showing a low amount of waxes and a high contamination level, had an opposite position. Brassicaceae accessions were divided into two clusters: the wild rocket accessions (24 and 32) were characterized by high fatty acid content, high hydrophobicity, and low *S. enterica* attachment, and all other accessions were less susceptible to *Salmonella* contamination and showed greater roughness. Chenopodiaceae formed a uniform cluster between Brassicaceae and Asteraceae (Fig. 4(B)).

Partial least squares results

The PLS model highlighted three components sufficient to describe the six variables considered (Fig. 5). The variance

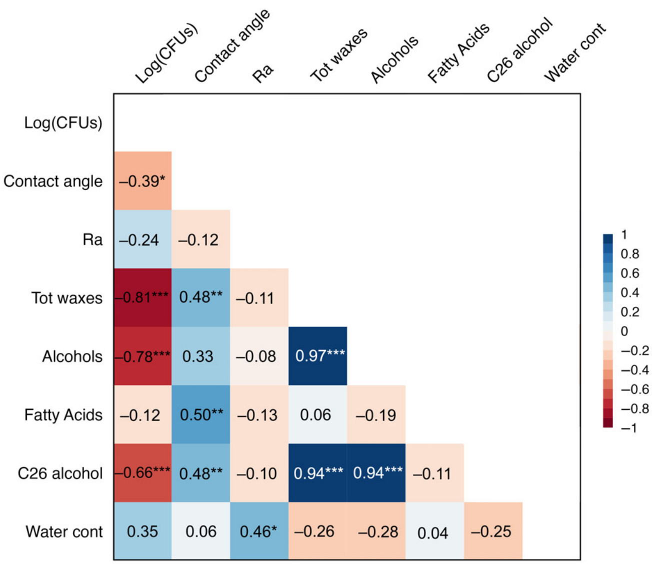

Figure 2. Pearson's correlation test ($P \leq 0.05$) showing the relationship between *Salmonella enterica* ATCC 19585 attachment and different leaf characteristics in 30 baby-leaf accessions. The heatmap represents the positive (blue) or negative (red) correlation. Significance levels are $P < 0.001$ ***; ≤0.01 **; $P \leq 0.05$ *; $P > 0.05$ is not significant.

explained by the model was 78.3%, and R^2 was 0.78 (values between 0 and 1, with 1 indicating a perfectly fit model) with a low root mean square error (RMSE) value (0.26) (this means that there is a good measure of how well the model predicts the response, lower values of RMSE indicate a better fit). The variables that showed a high importance for projection score (VIP) were: alcohols (1.53), C26 alcohol (1.33), contact angle (0.87) and water content (0.87). The same variables reported a coefficient of determination lower than 32% on the *S. enterica* attachment. In particular, the highest values were reported in alcohol ($R^2 = 0.31$) and C26 alcohols ($R^2 = 0.26$).

DISCUSSION

Numerous studies have documented varying degrees of vulnerability to human pathogen contamination in different types of vegetables.[11-13,33] Jacob and Melotto (2020)[11] found significant variations in the attachment and persistence of *S. enterica* and *E. coli* among 11 lettuce genotypes belonging to both *Lactuca sativa* (cultivated lettuce) and *L. serriola* (wild lettuce), with the latter species being less susceptible to contamination than the *sativa* group. In our study, differences in the attachment of *S. enterica* among 30 accessions of baby-leaf salads were observed and, in particular, wild lettuce showed the lowest level of contamination (Table 1). In a previous study,[12] these 30 accessions were analyzed for their susceptibility to *E. coli* attachment with similar results – that is, cultivated lettuce was more prone to *E. coli* attachment than its wild counterparts.

It is known that leaf traits can influence the behavior of human pathogens on vegetable crops. In this study, the hydrophobicity, leaf roughness, and epicuticular wax composition of the 30 baby leaves were investigated to identify the surface properties possibly associated with susceptibility to *S. enterica* attachment.

Leaf surface hydrophobicity can best be quantified by contact angle measurements. This is an indicator of the wettability of solid surfaces and ranges from 0° to 180°.[34] When it is 0°, the surface was completely wet, whereas, on the other hand, 180° corresponded to a completely non-wetting status; surfaces with contact angles greater than 90° are considered hydrophobic, while hydrophilic surfaces have contact angles less than 90°.[35] Among the 30 baby leaves, 76% were hydrophilic on the adaxial leaf surface (contact angle <90°), whereas seven of them (wild lettuce, wild Rocket 'Yeti' and Ingegnoli, mizuna, Rocket, Red chard 'Bull's Blood Artica', and Swiss Chard) had hydrophobic surfaces (contact angle >90°) (Table 1). In particular, wild lettuce, the accession least susceptible to contamination, showed also the greatest contact angle (135.54 ± 5.52°). Sorrel, the most susceptible, had a hydrophilic surface (contact angle = 46.76 ± 5.78°). Considering all the accessions, a significant negative correlation between

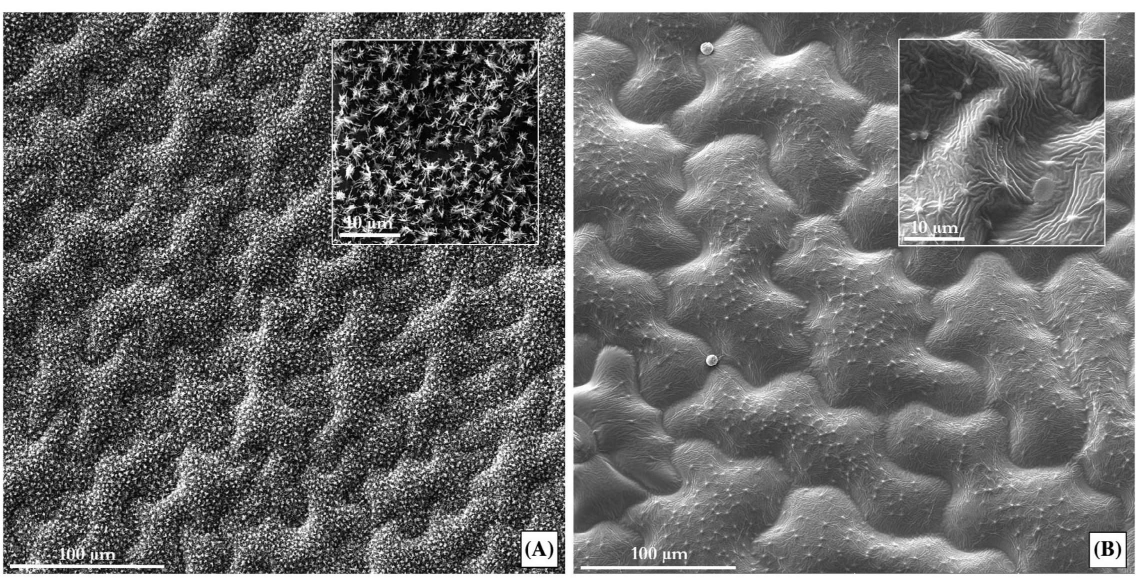

Figure 3. Scanning electron microscopy image (800×, and 8000× resolution) of adaxial leaf surface of wild lettuce (A), and sorrel (B).

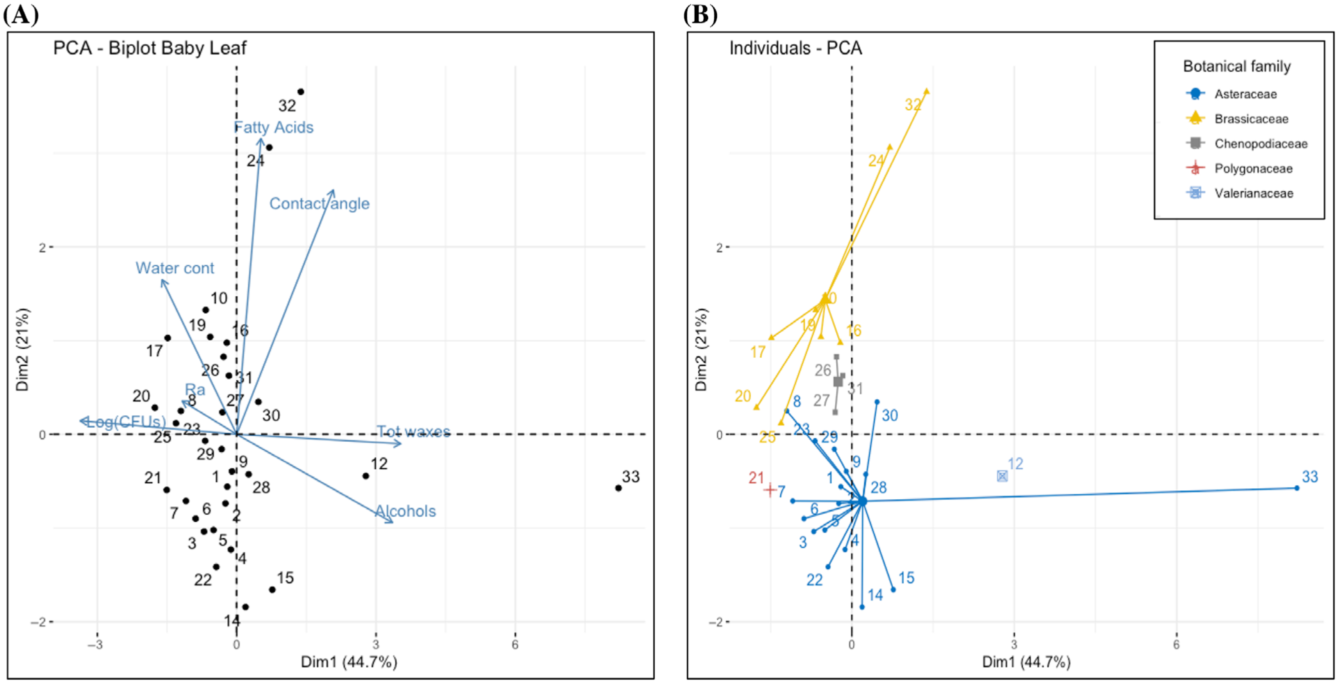

Figure 4. Principal component analysis (PCA) considering leaf characteristics (panel A) and individuals grouped in botanical families (panel B).

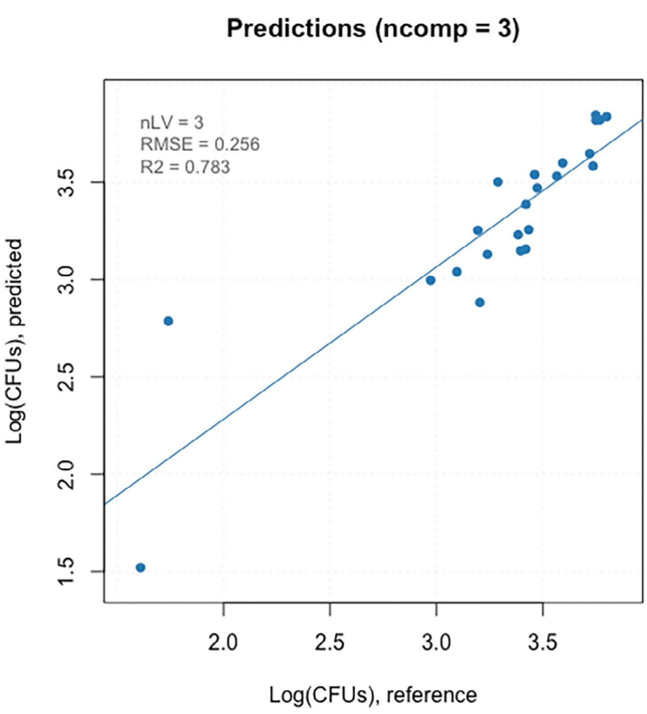

Figure 5. Partial least squares (PLS) prediction model for the amount of *Salmonella enterica* attachment using six variables (contact angle, surface roughness, C26 alcohol, alcohols, fatty acids, and water content).

attachment and the contact angle was observed (Fig. 2), supporting the findings of Hunter *et al.* (2015)[33] in lettuce.

The baby leaves also differed in roughness (Ra values) (Table 1). Consistent with Fig. 1, Red Giant leaf mustard showed the roughest leaf surface (Fig. 1(A)) whereas lamb's lettuce 'Trophy F1' had a smooth surface (Fig. 1(B)). Although the latter species also had one of the least contaminated accessions, the Pearson's test revealed that *S. enterica* attachment was not correlated to roughness. Considering that we adopted an incubation time of 5 min, this result suggests that roughness was not a decisive factor in the early phases of the contamination process. This short inoculation time was adopted in agreement with other authors,[15,36,37] to ensure that *Salmonella* attachment resulted solely from the surface wetting and to minimize the water absorption by the leaves. On the other hand, several authors[12,33,38] found that bacterial attachment was correlated positively with leaf roughness after longer surface inoculation (1, 1.5, and 2 h, respectively). In our study, roughness showed a slightly positive correlation with water content (Fig. 2). As far as the authors are aware, no previous study found this relationship. Turgidity given by the high water content can be hypothesized to increase the difference in height between bulges and hollows on the leaf surface, also increasing the roughness in turn.

Epicuticular waxes form the outermost layer of the leaf surface, which come into direct contact with human pathogens during plant contamination. Previous studies demonstrated that waxes can hinder *E. coli* and *S. enterica* attachment in different vegetable crops.[33,36,39] Similarly, rotavirus adsorption in 21 leafy greens was negatively correlated with total wax concentration, fatty acids, and alkanes.[40] In our study, a negative correlation between *S. enterica* attachment and the total waxes was observed (Fig. 2). According to Pearson's correlation test, the wax components most correlated with attachment were alcohols, the main constituent of the waxes in the 30 baby leaves, and in particular C26 alcohol, the most abundant alcohol found ($r = -0.78$, and -0.66, respectively). The surface images taken by SEM revealed that the epicuticular layers of wild lettuce leaves (the accession with the highest concentration of waxes) showed the crystalline structures typical of C26 alcohol, while sorrel (the accession with the lowest concentration of waxes) had smooth layers (Fig. 3).

Interestingly, the two accessions were also the least and the most susceptible to *S. enterica* attachment. This result suggests that the visible crystalline wax structures on wild lettuce leaves contributed to the low *S. enterica* attachment we observed in this species. These findings agree with those reported by Ku *et al*. (2020),[36] who found a lower *S. enterica* attachment associated with more abundant epicuticular waxes and C26 alcohol on adaxial leaf surface of lettuce 'Two Star'. Ensikat *et al*. (2011)[17] suggested that the 3D epicuticular wax crystal morphology influences the hydrophobicity of the leaf surface, as the size and the number of the crystalline facets are believed to reduce the contact area with water. In agreement with the study of Ensikat *et al*. (2011),[17] the current study noted a positive correlation between the contact angle and the C26 alcohol (Fig. 2). The hydrophobicity was also positively correlated with fatty acids (Fig. 2), similar to the observations of Lu *et al*. (2015).[40] That was not surprising considering that fatty acids are lipids containing long-chain hydrocarbons that end in a carboxylic acid functional group, thus being hydrophobic. It is known that fatty acids play a crucial role in defense against pathogens in plants.[41] Not only do they form physical and chemical barriers but they also activate defense signaling pathways when they come into contact with phytopathogens. In particular, C16 and C18 fatty acids contribute to defense regulating basal, effector-triggered, and systemic immunity of plants.[41] Both of these fatty acids were detected in the *Diplotaxis tenuifolia* accessions (wild rocket 'Yeti' and wild rocket Ingegnoli) (data not shown), which, among the 30 baby leaves, had the highest fatty acid content as shown in Table 2 and Fig. 4(A) (accessions 24 and 32). Wild rockets were also among the less contaminated accessions (Table 1). It can therefore be hypothesized that, in *Diplotaxis tenuifolia*, fatty acids were crucial in limiting *S. enterica* attachment. However, due to the short inoculation time, they probably exert their role by increasing leaf hydrophobicity more than by more complex mechanisms. Indeed, wild rockets also showed a large contact angle (Table 1), resulting among the hydrophobic accessions. Considering all the 30 accessions, the incidence of fatty acids was less decisive; in fact, the Pearson's test revealed a non-significant correlation between their content and bacterial attachment (Fig. 2). Thus, based on our results, fatty acids, as well as other factors, may have a predominant role in the susceptibility to *S. enterica* contamination depending on the species. In lamb's lettuce, which showed a level of contamination similar to wild lettuce (Table 1), the low attachment by *S. enterica* could perhaps be related to the presence of thymol, whose antibacterial effects are well known.[42] Such hypothesis is supported by Xu *et al*. (2008),[43] who found that thymol had a detrimental effect on *E. coli* due to its ability to permeabilize and depolarize the cytoplasmic membrane.

Based on the PLS model results, the alcohols, C26 alcohol, contact angle, and water content had the largest impact on the attachment of *S. enterica* in the 30 baby leaves in this study. Together, these variables can explain 78% of the variation in the bacterial attachment among the accessions. The highest coefficient of determination was observed between bacterial attachment and the concentration of alcohols ($R^2 = 0.31$) and C26 alcohol ($R^2 = 0.26$).

CONCLUSIONS

This study confirmed varying degrees of susceptibility to contamination by *S. enterica* among different leafy vegetables. Among the 30 baby-leaf salads investigated, the lowest attachment was found in wild lettuce (*Lactuca serriola*) and lamb's lettuce 'Trophy F1' (*Valerianella locusta*). The study also demonstrated that leaf surface properties influence attachment by this human pathogen. In all sets of baby leaves the crucial traits were leaf surface hydrophobicity (measured as contact angle) and epicuticular waxes. The most important wax components were alcohols and, in particular, the 3D wax crystals of C26 alcohol, which significantly increased hydrophobicity, especially in wild lettuce. In wild rocket accessions, *S. enterica* attachment was probably hindered by fatty acids due to their hydrophobic nature.

These findings can help predict and control the attachment and contamination of leafy salads by enterobacteria. They also provide useful information for breeding programs aimed at developing cultivars that are less susceptible to human pathogens and therefore safer.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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